



# The exceptionality of stress response in *Magnaporthe oryzae*: a set of “salt stress-induced” genes unique to the rice blast fungus

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**Abstract** The ability of pathogens to signal perception and adaptation to environmental changes is an important prerequisite for successful colonization of the host organism. Filamentous phytopathogenic fungi, for example, have to cope with rapid changes in the environment during invasive growth *in planta*. Consequently, they have evolved a range of specific factors contributing to environmental adaptation facilitating host invasion. In addition to conserved pathways, including genes participating in stress response, unique/individual genes within the pathogens might represent determinants of pathogenicity. Therefore, identification of unique genes could provide a set of excellent candidates for novel and specific fungicide targets. One of the environmental changes during host invasion comprises the accumulation of osmolytes, which are present in varying concentrations inside the plant. Transcriptional profiling of the rice blast fungus *Magnaporthe oryzae* undergoing osmotic stress revealed interesting results. We identified a set of 239 genes which were regulated significantly by salt stress. Among these “salt stress-

regulated” genes, 176 (75%) of the upregulated and all of the downregulated genes were found to have no homologues in yeast when interrogation against the yeast protein database was performed. Functional annotation analysis by InterProScan and clustering of genes based on gene ontology (GO) enrichment analysis was conducted to annotate each of the “salt stress-regulated” genes and to identify functional categories of biological processes associated with environmental stress response. Finally, we present a set of “salt stress-regulated” genes suggested as unique in the rice blast fungus.

**Keywords** NGS · Pathogenicity · Environmental stress · Fungicide target · Osmoregulation · *Magnaporthe oryzae* · Salt stress-induced genes

## Short communication

The transcriptome reflects differential gene expression patterns by defining a dynamic link between an organism’s genome and its physiological properties. We conducted a comprehensive genome-wide differential expression analysis (RNA-seq) regarding 0.5 M KCl stress in the filamentous phytopathogenic fungus *Magnaporthe oryzae* in order to find unique gene expression profiles under “host-mimicking” environmental stresses. Using salt stress as a host-mimicking agent appears to be feasible, because salt ion concentration of nearly 0.5 M for K<sup>+</sup> has already been documented in plant tissues [5, 6]. The resulting “salt stress-induced” genes provide a promising reservoir for novel fungicide targets, since osmoregulation of phytopathogenic fungi is already known to be a suitable target location for crop protection. The high osmolarity glycerol pathway is a druggable signalling pathway comprising

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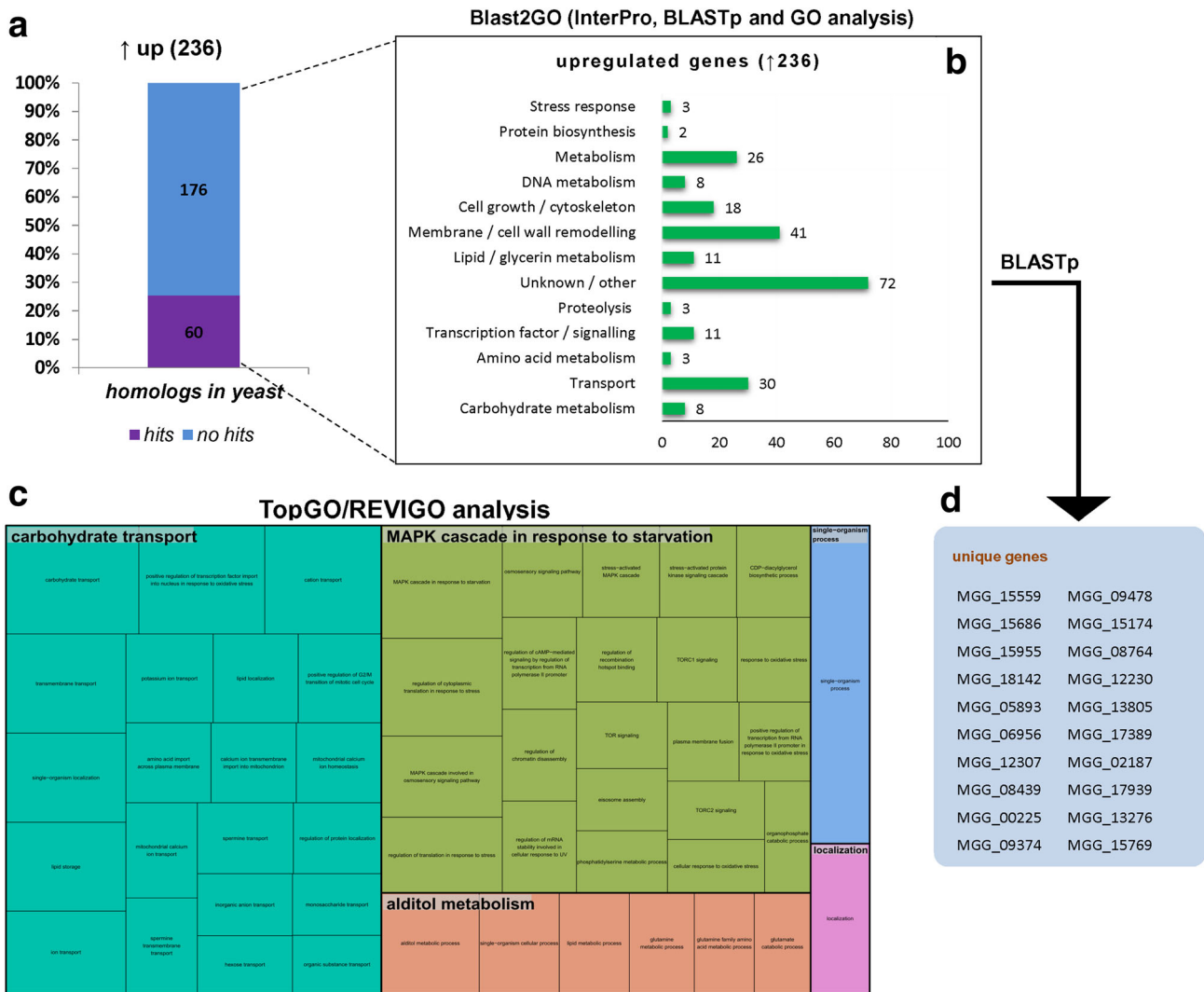
different target proteins [11, 13]. Thus, the identification of genes contributing to osmoregulation or genes responsible for adaptation to environmental stresses is of high interest for fungicide research.

We identified a total of 236 genes which were significantly upregulated and three genes downregulated in response to salt stress. Analysing the deduced protein sequence homology of these “salt stress-regulated” genes by interrogation of the *Saccharomyces cerevisiae* genome database ([www.yeastgenome.org](http://www.yeastgenome.org)) resulted in a set of 176 (75%) upregulated genes and all of the downregulated genes exhibited no assignable homology to proteins in *S. cerevisiae*. We focused on the representation of the 236 upregulated genes (Fig. 1a), but raw data and annotations for the three downregulated genes are provided in the supplementary information (Tab. S1). We checked whether some of the upregulated candidate genes for which the homologues in yeast were found are already known to possess functions in stress response, osmoregulation or even cellular signalling in *S. cerevisiae* to validate the data obtained from RNA-seq analysis. Hence, we identified several instances of well-fitted correlation with datasets published previously. We found that the genes MGG\_01822 (*MoHOG1*) and MGG\_10268 (*MoPBS2*) were significantly upregulated when subjected to salt stress, which is in accordance with functions published already regarding osmotic stress regulation either in yeast [9] or *M. oryzae* [4, 10]. Further evidence is given by genes predicted to encode homologues to the yeast ATPase *ENA2* (YDR039C), which is involved in salt tolerance [8, 16] or even a group of genes involved in stress-induced transport mechanisms (Tab. S1). The protein sequences of the “salt stress-induced” genes deduced were interrogated against the non-redundant database from the National Centre for Biotechnology Information and Pathogen Host Interactions (PHI) databases with the aim of identifying homologues in other organisms and putative pathogenicity-associated factors. The interrogation of the genes selected in the PHI database partly confirmed the hypothesis that the salt stress-affected genes identified may be related to pathogenicity-related processes in filamentous fungi. The PHI-based homology search resulted in 35 (of 236) listed homologues in the public database, and 22 (63%) of them have been published to be virulence or pathogenicity factors (Tab. S1). Furthermore, we addressed our hypothesis of a putative relationship between environmental stress and virulence by comparing the “salt stress-induced” genes with *Magnaporthe* differentially expressed genes during *in planta* growth/colonization as well as upon heat or drought stress condition (as published by [2, 12, 14]). As a result, we found an overlapping set of 43 genes regulated in at least two of these conditions, but none of the genes exhibited significant regulation in all of these conditions (provided in

Tab. S1, “conditionally regulated genes”). Consequently, these results indicate both functions of these genes in similar physiological networks and involvement in different signalling pathways. Addressing the physiological function of the unknown “salt stress-regulated” genes, we classified the candidates into different functional categories based on Blast2GO processing of the data, including BLASTp, InterPro and GO analysis. Thus, most of the gene products predicted could be assigned to such functional categories as metabolism (26), membrane/cell wall remodelling (41) and transport (30). A functional annotation for 72 of the differently expressed (DE) genes was, however, not predictable (Fig. 1b). The GO analysis was completed with an enrichment analysis to extract the maximal meaning of the RNA-seq data. Further analysis using TopGO/REVIGO refined these findings, because the REVIGO treemap displays GO terms matching biological processes which are appropriate for cellular response to salt stress (Fig. 1c). As a conclusion, all these data enabled us to present an exceptional list of 20 *Magnaporthe oryzae*-unique “salt stress-upregulated” genes having no homologues in other fungal genomes listed in public databases (Fig. 1d). The set of specific genes will initialize future projects studying the function of osmoregulation and appear to be promising candidates for finding novel fungicide targets for plant protection.

## Methods

Conidia of *M. oryzae* were collected from eleven-day-old complete medium agar plates [11] and adjusted to  $10^5$  conidia/ml. Spore suspensions were used as inoculum for liquid complete medium and incubated for 48 h at 28 °C at 100 rpm. These cultures were used to inoculate the main axenic cultures, which were cultivated for a further 24 h at 28 °C at 100 rpm. Total RNA from three biological replicates was extracted as an untreated control using the RNeasy Mini Kit (QIAGEN, plant protocol). Subsequently, RNA was extracted from the same cultures after 25-min treatment with 0.5 M KCl. Prior to transcriptome sequencing, equal amounts of total RNA derived from three biological replicates for each condition (untreated and 25 min after KCl induction) were pooled together. Library preparation was carried out using 1.5 µg of total RNA for each. The RNA-Seq readings were obtained from a  $2 \times 100$  bp paired-end Illumina HiSeq 2000 single lane run for each condition investigated, followed by a quality check using Qualimap version 2.2 [7] and removal of adaptor sequences. The readings retained were merged using FLASH 1.2.9 (FLASH source: <http://sourceforge.net/projects/flashpage/?source=dlp>) and aligned to the reference genome of *Magnaporthe oryzae* 70-15 version 8



**Fig. 1** Illustration of the analysis of the 236 “salt stress-upregulated” genes in *Magnaporthe oryzae*. Blast2GO analysis (InterPro, BLASTp and GO analysis) was conducted with the 236 candidate gene sequences to refine/update the existing annotation. **a** Bar chart representing potential homologues in yeast, obtained from sequence interrogation in yeast databases of significantly expressed genes regarding salt stress (25 min, 0.5 M KCl). **b** Customized functional gene categories of upregulated genes. Most of the differentially expressed genes were found to be involved in metabolism, membrane/cell remodelling or transport-associated processes. **c** REVIGO

treemap summarizing GO biological process categories overrepresented regarding salt stress. TopGO was used to identify GO biological processes that were overrepresented among transcripts with increased abundance upon KCl-response at  $p$ -values of  $\leq 0.05$ . Similar colours indicate semantic similarity related to similar functional category. The size of each rectangle is proportional to the  $p$ -value for that category. **d** A set of 20 *Magnaporthe oryzae*-unique genes upregulated regarding salt stress (25 min, 0.5 M KCl) for which no homologues in other fungal species were found in public databases

obtained from the Broad Institute by employing TopHat 2.0.11 [18]. Expression levels for each gene were determined by using the CuffDiff quantification method [18], and reading counts were expressed as FPKM (“Fragments Per Kilobase of transcript per Million fragments mapped”) values. Only those genes, having at least a fivefold change and displaying the FPKM value of at least ten in any of the conditions investigated, affirmed by a  $p$  value of  $\leq 0.05$ , were considered as significantly differentially expressed and were selected for further analysis. The BLASTp search against the National Centre for Biotechnology Information

non-redundant database was conducted to identify functional annotation orthologues in other fungal organisms, using the taxonomy filter “fungi” (taxa 4751, Fungi) and an  $E$ -value threshold of  $1.0E-3$ . Furthermore, a local BLASTp interrogation (using  $1.0E-30$  as an  $E$ -value cut-off) against the *Saccharomyces cerevisiae* (S288C) protein sequences obtained from the *Saccharomyces cerevisiae* genome database was performed to examine the proportion of corresponding DE genes. Additionally, gene ontology and functional annotation of the protein sequences deduced were employed using Blast2GO 4.0.2 [3] via searching for

best hits with the following cut-offs: *E*-value 1.0E-6 and an internal annotation cut-off value of 55, to classify genes into GO terms based on molecular function, biological processes and cellular components. The results from the BLASTp analysis, functional domain comparisons from InterProScan (restricted to BlastProDom, HMMPfam and TMHMM) implemented in Blast2GO and the prediction of signal peptides with SignalP 4.1 software [15]; <http://www.cbs.dtu.dk/services/SignalP/>), as well as interrogation against the PHI database (version 4.0) were used to make final assignments to GO functional categories of each gene, indicated in the final table as “customized category”. Finally, GO enrichment (by focusing exclusively on the biological process) of DE genes was computed using the TopGO package (release version 2.26.0; [1] implemented in Bioconductor 3.3, employing Fisher’s exact test for statistical significance to extract the maximal meaning of the RNA-Seq data. The enriched GO terms at *p*-values of  $\leq 0.05$  were retained and then slimmed in REVIGO [17]; <http://revigo.irb.hr/>) by removing redundant GO terms. Treemaps were produced to visualize enriched and non-redundant GO categories. A total of 130 of the “salt stress-regulated” genes were not suitable for GO enrichment analysis, because they represented hypothetical proteins or displayed no identity to other proteins with assigned GO terms in the database. The total list of DE genes obtained from RNA-seq analysis, containing comprehensive information regarding functional signatures for each gene, is provided in Table S1. GO annotation obtained from JGI (<http://genome.jgi.doe.gov/Maggr1/Maggr1.home.html>) is also provided in Table S1 for augmented comparative analysis.

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